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THE GROWTH OF *PROTEUS* ON AMMONIUM LACTATE *PLUS* NICOTINIC ACID.

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IT is generally recognized that colon bacilli will in most cases grow satisfactorily on some simple medium such as ammonium lactate with the usual salts. For instance all of 6 recently isolated strains grew well in 24 hours, but one which had been maintained for a long time on trypsin-hydrolysed casein grew only when amino-acids were supplied. In no case was the addition of an accessory vitamin necessary. On the other hand reports on the growth requirements of *Proteus*, summarized by Knight (1936), are conflicting. Some authors have claimed that all nitrogen requirements can be satisfied by NH_3 or amino acids, while others have failed to obtain continued growth under these conditions.

In the present work no growth of 10 strains of *Proteus* capable of serial subculture in the same medium was obtained either in ammonium lactate or in a full mixture of amino-acids such as that used by Fildes, Richardson, Knight and Gladstone (1936) for the growth of *Staphylococcus*. Good growth however followed when mere traces of such materials as peptone were added to ammonium lactate and it appeared probable that a vitamin was involved. A test of known vitamins showed that nicotinic acid was the essential requirement.

Medium used

KH_2PO_4	4·5 g.
$(NH_4)_2SO_4$	0·5 ,,
NH_4Cl	0·5 ,,
M/2 Na lactate	50·0 c.c.
Water to	900 c.c.

The pH was adjusted to 7·6, the mixture tubed in 9·0 c.c. lots and autoclaved. After autoclaving the following solutions were added to each tube by hand :

Ferrous ammonium sulphate $M/500$ in $N/50$ HCl (filtered)	0·25 c.c.
$MgSO_4$, $7H_2O$. 0·4% in water (autoclaved) . . .	0·1 ,,
Water to a final	10 ,,

The materials used were all of analytical quality except the lactate. The only satisfactory preparation of lactate for growth purposes was obtained from zinc lactate recrystallized 3 times. From this the lactate was recovered as the sodium salt by means of sodium carbonate. All customary precautions were taken against the introduction of unsuspected chemical contaminations. From a personal communication from Prof. W. W. C. Topley it appears that distilled water can contain traces of copper sufficient to inhibit growth of *Bact. aërtrycke* under these conditions. The water used in this laboratory is obtained from a copper still heavily tinned and is satisfactory. Nicotinic acid was used as an autoclaved 10^{-3} M solution, 0·2 c.c. being added per tube making 2×10^{-5} M in the culture.

Strains tested

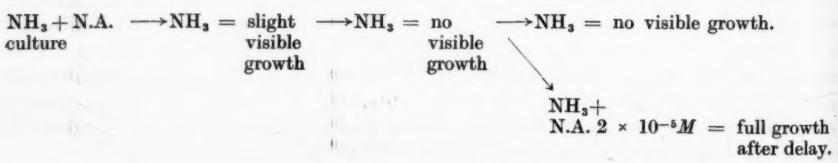
10 strains were examined ; 1 strain of X 19, an old laboratory culture ; 4 strains from urine ; 3 strains from fæces ; 1 strain from the throat and 1 strain from an anaërobic abscess. All except X 19 were recent isolations. When grown in the presence of added tryptophane X 19 produced indole but the others all failed.

Procedure

Mixtures were made in test tubes and inoculated. The contents of the tubes were then poured into 50 c.c. Erlenmeyer flasks and incubated in air + 5% CO_2.

The cultures started from agar, a needle point being transferred to the mixture containing nicotinic acid (N.A.) Growth occurred with all strains in 24 hours and became heavier on the second day. One drop of each culture was transferred to the same medium and successfully propagated for 9 generations.

The following subcultures were then made, 1 drop (approximately 1/20 c.c.) being transferred in each case :


NH₃ + N.A. culture
NH₃ = slight visible growth
NH₃ = no visible growth
NH₃ = no visible growth.
NH₃ + N.A. 2 × 10⁻⁵M = full growth after delay.

Thus all strains of *Proteus* tested grew visibly in serial subculture on NH_3-lactate + nicotinic acid, while none would grow visibly after one subculture in the absence of nicotinic acid. The fact that viable bacteria were still present in cultures without visible opacity was clearly due to a subvisible growth due to transferred traces of nicotinic acid.

The quantitative relationships of nicotinic acid compounds to growth of Proteus.

Tests were made of the activity of 3 related compounds, namely nicotinic acid, nicotinic acid amide and cozymase (adenine-ribose-phosphate-nicotinamide) by means of serial dilutions added to the basal medium. Nicotinic acid and the amide were dissolved in water and cozymase in phosphate buffer pH 6·0, all in a concentration 10^{-4} *M*, and sterilized by Seitz EK filtration. An experiment showed that none of the activity was adsorbed by the filter. The inoculum was obtained from the centrifuged deposit from a culture of *Proteus* which had been grown in the basal medium containing a suboptimal ($2 \times 10^{-8}M$) concentration of nicotinic acid. The deposit was washed once in distilled water and diluted in the same to invisibility. One drop was added per tube (10 c.c.). The basal medium in all quantitative work was measured into the tubes after sterilization.

This experiment, and others similar, showed that the activities of the 3 compounds were the same, the smaller activity of the acid found by Knight (1937*a*) in the case of the staphylococcus not being demonstrable. Full growth under the conditions used was obtained in 2 days with $2{\cdot}5 - 1{\cdot}25 \times 10^{-6}M$. Less than this concentration led to a diminished mass of growth, $10^{-8}M$ giving only a trace. Since however the mass of growth depends, at the limit of the titration, on the quantity of nicotinic acid present, it will be understood that an " end point " is purely arbitrary and growth may be shown to have taken place far beyond the limits of visibility.

The fate of nicotinic acid in a culture.

Since the mass of growth is governed over the suboptimal range of titrations by the amount of nicotinic acid present, it follows that in this range no nicotinic acid should be left free. This was found to be the case.

Experiment 1 was carried out with nicotinic acid in the same manner as previously. It is seen that $2{\cdot}5 \times 10^{-6}M$ gave full growth whilst half this concentration gave slightly less. After 2 days the cultures were filtered (Seitz EK), diluted with an equal part of the medium and reinoculated. The subsequent growth showed that nicotinic acid was no longer present in filtrates from cultures in which the amount of added nicotinic acid was suboptimal. Thus the whole of the nicotinic acid which was required for growth had been removed, only that remaining which was in excess of requirements.

EXPERIMENT 1. *Removal of nicotinic acid in growth.*

Titration of nicotinic acid

		Molar concentration of nicotinic acid.	Growth in 2 days.
Flask 1	Medium +	0	0
2	+	5×10^{-6}	++++
3	+	$2{\cdot}5 \times 10^{-6}$	++++
4	+	$1{\cdot}25 \times 10^{-6}$	+++⊥
5	+	$6{\cdot}25 \times 10^{-7}$	|
6	+	$3{\cdot}12 \times 10^{-7}$	|
7	+	$1{\cdot}56 \times 10^{-7}$	|
8	+	$7{\cdot}8 \times 10^{-8}$	tr

Titration of filtrates

			Growth in 2 days.
Flask 1	Medium +	0	0
2	+	N.A. $5 \times 10^{-6}M$	++++
3	+	filtrate tube 2	+++
4	+	,, ,, 3	tr
5	+	,, ,, 4	0
6	+	,, ,, 5	0
7	+	,, ,, 6	0

Plus signs roughly in proportion to the mass of growth. tr = trace.

Synthesis of cozymase.

It has been shown by Lwoff and Lwoff (1937*a*) that Warburg's coenzyme or cozymase can replace and is presumably identical with the active "V" factor required by *H. influenzæ* or *parainfluenzæ*. This demonstration was among the considerations which led Knight (1937*a*) to the identification of nicotinic acid as one of the constituents of his "staphylococcus factor" and it was assumed that the necessity for the supply of nicotinic acid was due to its requirement in the synthesis of cozymase by the bacterium (Fildes, 1937; Knight, 1937*b*). The same assumption may be made in the case of *Proteus*. In the case of *staphylococcus* grown in peptone water the production of "V" factor (cozymase) may be shown by filtering the grown culture and inoculating the filtrate with *H. parainfluenzæ* or, with the addition of hæmatin, *H. influenzæ* (*vide* Fildes, 1923 with contemporary literature). If growth takes place cozymase must be present. A similar procedure was used in the case of *Proteus*.

Experiment 2.—Cultures were arranged in the NH_3-lactate medium containing nicotinic acid in excess ($10^{-5}M$) and allowed to grow for 4 days. The cultures were then filtered and tested for cozymase. The medium used for the growth of *H. parainfluenzæ* as the test object for the presence of cozymase was the peptone-glucose-buffer medium used by Lwoff and Lwoff (1937*a*).

The inoculum was 1 drop of a culture of *H. parainfluenzæ* (NCTC 4101) grown on the Lwoff medium + 0·2 c.c. cozymase $10^{-4}M$ in 10 c.c., and diluted with water till only just visible.

EXPERIMENT 2. *Synthesis of cozymase.*

	Quantities in c.c.	Growth in hours. 21	43
Tube 1	Lwoff medium 5·25 + NH_3-lactate medium 4·25 + water 0·5	0	0
2	„ „ + NH_3-lactate medium 4·25 + nicotinic acid $10^{-4}M$ 0·5	0	0
3	„ „ + NH_3-lactate medium 4·25 + cozymase $10^{-4}M$ 0·5	++++	++++
4	„ „ + Proteus-filtrate 4·25 + water 0·5	+	+
5	„ „ + Proteus-filtrate 4·25 + cozymase 0·5	++++	++++

Experiment 2 shows that growth took place in the presence of the *Proteus* culture in place of cozymase, indicating that cozymase had been synthesised and discharged in small quantities into the surrounding medium. A similar experiment carried out with *Bact. coli* growing on the same medium but without nicotinic acid gave also a positive result showing that cozymase is associated with the growth of *Bact. coli* also but that this substance can be synthesised from NH_3.

DISCUSSION.

Nicotinic acid compounds have been shown to be necessary additions to the nutrients of *Staphylococcus* (Knight, 1937*a*), *C. diphtheriæ* (Mueller, 1937), dysentery bacillus (Koser, Dorfman and Saunders, 1938) and now of *Proteus*. Since cozymase is shown to be synthesised during the growth of *Proteus* with nicotinic acid or of *Bact. coli* without, it may be assumed that cozymase or possibly some other similar pyridine nucleotide is a necessary reagent in the growth of these organisms or indeed of most organisms since most are capable of inducing the satellite phenomenon in the growth of *H. influenzæ*.

It is generally understood (*cf.* Green and Brosteaux, 1936) that pyridine nucleotides (cozymase) are in some cases necessary intermediates in the chain of reactions by which hydrogen is transferred from an oxidisable substance (lactate) to oxygen or some other hydrogen acceptor. The hydrogen activated by a dehydrogenase is transferred to oxidised cozymase and this reduced substance is then reoxidised by hydrogen transfer to other transporter systems connecting with the final hydrogen acceptor. Such systems may in some cases be the flavin system of Warburg or the cytochrome system of Keilin. According to Green and Brosteaux (1936) animal lactic dehydrogenases require the cozymase link and then the process continues through the flavin system. On the other hand the work of Lwoff and Lwoff (1937*b*) indicates that the dehydrogenase of *H. parainfluenzæ* also requires the cozymase link (V factor), but that

the subsequent hydrogen transfer to oxygen proceeds through Keilin's system (X factor). It may be inferred that pyridine nucleotides function as codehydrogenases in the case of other bacteria but the subsequent path of hydrogen transfer in these cases is not yet certain. Yudkin (1937) has indeed shown that cozymase is necessary in the dehydrogenation of lactate by *Bact. coli*.

Variation in the power of different species to synthesise pyridine nucleotides accounts for their varying nutritive requirements. Though *H. influenzæ* and *parainfluenzæ* may be supplied with the pyridine and nucleotide fractions separately they are unable to combine them (Lwoff and Lwoff, 1937*a*). On the other hand *Staphylococcus* and *Proteus* are unable to synthesise the pyridine fraction but can combine it, when supplied with it, with the nucleotide. Other organisms such as *Bact. coli* can synthesize both fractions and combine them and so require no " accessory " addition of nicotinic acid to their diet.

CONCLUSIONS.

1. Nicotinic acid is an essential accessory nutrient for 10 strains of *Proteus*.
2. It is the only nitrogenous substance required in metabolism which cannot be synthesised from NH_3.
3. Its function is to supply material for the synthesis of pyridine nucleotides.

My thanks are due to Dr. B. C. J. G. Knight and Mr. G. D. Greville who prepared the cozymase used in this work, to Dr. B. W. Lacey who supplied me with the cultures, and to Professor James McIntosh for the facilities enjoyed by this department in the Bland-Sutton Institute.

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DISAGGREGATION OF PROTEINS BY ENZYMES.

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DURING the course of experimental work carried out on the relative degrees of denaturation of pseudoglobulin protein and of the diphtheria antitoxin associated with it, at varying pH values and temperatures, certain observations were made which appear to have importance both for the understanding of the chemistry of proteins, and as the basis of a method for securing a further degree of purification of antitoxins.

The pseudoglobulin solution* used for the early experimental work was obtained from the routine serum concentration process in the following manner: The precipitate, consisting of fibrin, euglobulin and some pseudoglobulin, which is produced by precipitation of diphtheria plasma at 30 per cent. saturation with ammonium sulphate, was collected, and the antitoxic pseudoglobulin contained in it extracted by solution and reprecipitation with ammonium sulphate. This low-grade antitoxic material was available in reasonable quantity and was therefore used for experimental work.

After dilution to approximately 2 per cent. protein content, and the addition of 5 per cent. NaCl, solutions were adjusted to varying pH values and heated at temperatures sufficient to produce protein denaturation. These varied from about 58–65° C., depending on the pH value of the solution. Samples removed after varying times were filtered and the filtrates examined. The antitoxin content was measured by the flocculation method (checked by animal tests), and the protein content by means of a Zeiss dipping refractometer.

Where these mixtures had been adjusted to pH values around 4·0, it was found that the destruction of antitoxin was much less than the denaturation and loss of solubility of protein, as a result of heating at certain temperatures. One experiment illustrating this phenomenon is given below:

The 2 per cent. pseudoglobulin solution, containing 5 per cent. NaCl and adjusted to pH 4·2, was heated in a water-bath at 60° C. for the periods shown in Table I. After filtration, the residual antitoxin and protein was determined and is shown as a percentage of the original amount. The number of antitoxic units associated with each gramme of protein is shown, together with the ratio of this figure to the original value, for each filtrate.

* This crude solution contained proteins other than the pseudoglobulin (euglobulin, etc.). The antitoxin referred to in this paper was diphtheria antitoxin, but similar results have been obtained with other antitoxins.

TABLE I.

Minutes in water-bath at 60° C.	Percentage protein left.	Percentage antitoxin left.	Units/gm.	Ratio.
0	100	100	7,150	1·0
5	75·2	93	9,100	1·23
10	53·4	84·5	11,600	1·58
15	26·6	70·5	19,500	2·65
20	17·0	59·5	25,000	3·50
25	15·7	59·5	27,200	3·80

By means of this heat treatment, under the correct conditions, it was found to be possible to secure a five-fold increase in the purity of the antitoxin for a reasonable yield of antitoxin.

When, however, an attempt was made to prove that this observation could be applied generally to pseudoglobulin solutions, it was found that this was not the case. A pseudoglobulin solution obtained directly by fractionation between the levels 30 to 50 per cent. saturation with ammonium sulphate showed quite different results when subjected to the same treatment as shown in Table II.

TABLE II.

Minutes in water-bath at 59° C.	Percentage protein left.	Percentage antitoxin left.
5	80·4	92·5
10	55·2	69·5
15	21·1	21·0
20	14·9	16·4
30	5·9	6·7

With this material, as will be seen from the table, denaturation of protein and loss of antitoxin proceeded at the same rate and there was no increased purity of the antitoxin.

Further work confirmed both these observations in that the antitoxic pseudoglobulin recovered from the 30 per cent. precipitation showed results of the type shown in Table I, while the pseudoglobulin from the 30–50 per cent. precipitation gave results as shown in Table II.

Eventually, the difference between these two types of pseudoglobulin materials was traced, and it was found that the difference in their response to heat denaturation lay in the fact that in one case the pseudoglobulin had been acted upon by the enzyme fibrinolysin, whilst in the other no such action had occurred.

This enzyme has been extracted from fibrin clot by means of dilute acetic acid, or alternatively the fibrin clots allowed to autolyse in dilute tricresol solution (0·2 per cent.) at 37° C. In either of these forms it has been found that the enzyme is capable of producing a change in the properties of a pseudoglobulin protein solution, so that it behaves in a differential manner on heat treatment under the conditions already described. In its action on a pseudoglobulin solution this enzyme does not appear to cause extensive protein

hydrolysis, as is shown by the fact that the amount of heat-coagulable protein present, before and after the enzyme has produced its effect, is the same. When this enzyme acts on solid fibrin, it converts a solid protein into a soluble form which shows all the properties of soluble non-denaturated proteins, *i. e.* heat coagulation at the iso-electric point, and reversible precipitation with salts such as ammonium sulphate. This autolytic activity of fibrinolysin is very much enhanced by the presence of small quantities of tricresol, and is a matter which will be dealt with more fully in other papers.

The specific action which fibrinolysin exerts on pseudoglobulin makes quite clear the difference in properties which was shown by the two classes of pseudoglobulin solutions. In one case the material was obtained by extraction from a precipitate which consisted largely of fibrinogen and fibrin, and which contained tricresol as an antiseptic. Under these conditions a certain amount of autolysis of the fibrin occurs, and the soluble protein produced, together with fibrinolysin, passes into solution with the pseudoglobulin, and the enzyme is therefore present in this product. Where, however, the pseudoglobulin is obtained directly between the levels 30–50 per cent. saturation with ammonium sulphate, only traces of the enzyme are present—too little to show any marked activity. That there is no other difference in the pseudoglobulin obtained between 30–50 per cent. saturation is shown by the fact that after treatment with fibrinolysin it behaves to heat treatment exactly as does the pseudoglobulin extracted from the 30 per cent. precipitation.

The filtrates obtained after heat treatment of pseudoglobulin solutions which have been acted upon by fibrinolysin contain both protein and antitoxin. The protein may be denaturated and rendered insoluble at higher temperatures than those used in the differential heating, and with this protein denaturation there is great destruction of antitoxin.

In view of the action of fibrinolysin on pseudoglobulin it became a matter of interest to find whether other better known proteolytic enzymes could produce a similar change in these antitoxic pseudoglobulin solutions, which would render them suitable for treatment by this method of fractional heat denaturation. Obviously a clear distinction must be drawn between this limited enzyme action and the well-known proteolysis involving the production of non-protein fragments, since these are not coagulated by heat and would not be removed by the method employed. If real hydrolysis of the protein takes place, it is no longer possible to remove the bulk of inactive non-antitoxic protein by fractional heat-coagulation methods.

So far all the proteolytic enzymes tried have given successful results, provided they are employed under correct conditions. These conditions vary for each enzyme, and will be dealt with in other papers.

The nature of the changes which take place when the pseudoglobulin solution is heated under the experimental conditions, before enzyme action has taken place, is shown by the following experiment:

A pseudoglobulin solution (diphtheria antitoxin) was prepared from the 30–50 per cent. sulphate fraction with a protein content of about 7 per cent. This was adjusted to a pH of 4·3 with citric acid, and heated in 50 ml. portions at 58° C. for 1 hour. The protein solution set to a solid coagulated gel. To each 50 ml. portion 200 ml. of water was added and the mixture well shaken.

One sample was filtered; the filtrate contained 9·5 per cent. of the original antitoxin and too little protein for estimation. A second sample (250 ml. of suspension) was treated with 1·0 gm. of pepsin, and then filtered as rapidly as possible. Its filtrate contained 40·5 per cent. of the original antitoxin. Similar samples treated with pepsin were incubated at 37° C. for 2, 24 and 48 hours before filtering. The antitoxin recovered in the filtrates is shown in Table III. With the return of the antitoxin in a soluble form in the filtrate there also appeared heat-coagulable protein. After such drastic treatment of the protein solution it is surprising to find that nearly 80 per cent. of the antitoxin could be recovered by enzyme treatment of the coagulated material.

TABLE III.

Treatment.	Protein in filtrate (heat-coagulable).	Percentage of original antitoxin found in filtrate.
None (control dilution) . .	++++	100
1 hr. at 58° C. pH 4·3* . .	trace	9·5
Plus pepsin and then:		
Filtered at once . . .	+	40·5
2 hrs. at 37° C. . . .	++	66·6
24 hrs. at 37° C. . . .	+++	78·9
48 hrs. at 37° C. . . .	+++	78·6

* This pH value is only that used for the particular experiment described. In routine work the desired enzyme action is obtained at very much lower pH values.

The re-solution of antitoxic material as a result of enzyme action depends on the degree of denaturation which has been produced, for if denaturation is produced by the use of higher temperatures, or lower pH values, or by such denaturating agents as alcohol, acetone or similar materials, then the action of proteolytic enzymes does not lead to the formation of a soluble antitoxic material. Further proof of this was found by subjecting antitoxic material which had already been liberated from the insoluble form by enzyme action to further heat or alcohol denaturation; enzyme treatment no longer gave soluble antitoxic protein from this product.

We were therefore led from these and many other experiments to picture the intact pseudoglobulin molecule as one having two protein components, one non-antitoxic with a low denaturation temperature, and the other bearing antitoxic properties and less easily denaturated. Critical denaturation under specific conditions denatures the first of these and renders the whole molecule insoluble, but by means of enzyme action the undenaturated portion may be split off, as a soluble protein still having its specific antitoxic activity.

If instead of allowing the proteolytically active enzymes to act on the material which has been rendered insoluble by partial denaturation, the enzyme acts on the undenaturated protein, then for a degree of hydrolysis which cannot be detected as a change in the total protein content a change is produced, which allows the easily denaturable portion to become insoluble without interference with the other antitoxin-bearing fraction. There is, however, considerable doubt as to whether any of the enzymes studied really

act on completely undenaturated protein. In most cases the conditions required for enzyme actions are such that this easily denaturated portion of the pseudoglobulin molecule is actually undergoing denaturation (although not necessarily becoming insoluble).

In considering these results we are, of course, dealing with the behaviour of the antitoxic-pseudoglobulin present in a crude solution of pseudoglobulin. The question whether " normal " pseudoglobulin exists in complex form, with two components, one easy and the other more difficult to denaturate cannot be answered completely. When " normal " pseudoglobulin is submitted to partial enzyme action, and then to heat-denaturation treatment, very little protein remains in solution. It is questionable whether any pseudoglobulin solution can be regarded as " completely normal " in the sense that it is free from any trace of antibodies owing to " natural immunity " which may be acquired by an animal. The trace of protein which survives the treatment may therefore be due to such antibodies, and the special properties of " antibody pseudoglobulin " may not be the same as those of " normal " pseudoglobulin. Nevertheless, there seems to be clear evidence that after hyperimmunization of an animal there exists in the pseudoglobulin fraction a considerable amount of material having no specific antitoxic properties which does appear to consist of a similar " two-part complex ". If the special properties were possessed only by the specific antitoxic-pseudoglobulin, it would be expected that sera from horses at different titres would all give a purified product of approximately the same unit/gm. protein value. This is not the case. Serum from a horse which after immunization has a value of 500 units/ml. will not give such a high figure for units/gm. protein as will a horse which after immunization produces 1500 unit/ml. serum. It is possible that the horse which fails to give high value antitoxic serum may, however, have produced a new protein complex which, while having the special properties of the antitoxic protein as judged by its " heat denaturation " properties, yet lacks the specific property of combining with toxin.

If the supposition of a complex molecule having two distinct properties is correct, there should be evidence for this belief. Some evidence is provided from experiments on specific precipitation of the antitoxin by its toxin,* and it has been shown that the amount of protein specifically precipitated is less in the case of " disaggregated antitoxin " than it is for antitoxin in its original form. The figure is approximately half, but this is not yet fully confirmed. Other evidence, obtained from ultrafiltration experiments,† shows that the modified antitoxin will pass through pores of a size which retain the original antitoxin. This result suggests that the modified form of antitoxin has a size of about one-half to one-third of that of the original material.

Still clearer evidence is available when the two forms of antitoxin are compared chemically, for it is found that a marked shift in pH-temperature stability exists between the two forms. There is also great difference in the extent to which the two forms of antitoxin can be adsorbed by aluminium hydroxide. The original form can be adsorbed almost completely, but this

* With Miss M. Healey (unpublished). See also Pappenheimer and Robinson (1937).

† In conjunction with Dr. Oakley.

property is lost as soon as the molecule is split into its two components by enzyme action.

In recent years the work carried out at Upsala has shown that certain proteins tend to become unstable at the extremes of their pH stability range, and may dissociate or disaggregate into units of half the molecular weight. Tiselius and Gross (1934) showed that horse hæmoglobin tended to dissociate on dilution, and Pedersen (1936) showed that interaction with certain other proteins tended to produce dissociation. These changes were not generally accompanied by irreversible denaturation, but Steinhardt (1938), working on the dissociation produced by amides, states that removal of these substances by dialysis may lead to reassociation, not in an orderly way, but in large molecular aggregates. The possibility of partial denaturation of the molecule is considered by Steinhardt, who quotes Sørensen's term of " incipient denaturation ". These observations on the altered state which may be induced in certain protein molecules in varying ways do not fall into line with the change produced enzymatically, for on one hand, there is little evidence that the smaller molecules differ in their properties from those of the larger, and on the other, that the two protein fragments derived by enzyme action from the original do differ markedly in their properties. One fragment has, in fact, a higher coagulation temperature and appears to carry all the specific antitoxin properties.

The first action of these proteolytic enzymes would appear to be that of specific disaggregation of large protein molecules into units which still possess the properties of proteins. Generally, under the conditions of enzyme hydrolysis, this stage is not very marked, since it is masked by the high velocity with which these protein fragments are reduced to a non-protein condition. At pH values away from the optimum for the enzyme it is more easily observed. Particularly is it true for fibrinolysin which can convert solid fibrin into a protein solution, showing heat coagulation, with little production of non-coagulable products.

It seems clear, therefore, that a protein molecule may give rise to protein fragments of smaller weight in two ways : either through simple dissociation into units of similar properties (a change which appears to be reversible), or through partial enzyme action into fragments which may have different properties (a change which appears to be irreversible). Probably not all proteins will yield protein fragments to enzyme action, just as not all proteins will undergo simple dissociation. Horse albumin, for example, does not appear to give such fragments.

In view of the general opinion that proteolytic enzymes act as catalysts and accelerate the rate of a reaction, numerous attempts have been made to produce this disaggregation without the aid of enzymes, but so far these have met with no success.

Interesting as these observations are, as bearing on the chemistry of proteins and of antitoxins in particular, it is perhaps of even greater interest and practical importance that these modified antitoxins of smaller molecular size show special properties when used for the purpose for which they are produced, that is, the treatment and cure of diseases. These new properties cannot, however, be dealt with in this paper and will form the subject of other papers.

SUMMARY.

In this preliminary paper the action of fibrinolysin in producing a change in the antitoxic pseudoglobulin molecule, so that it becomes disaggregated into protein components having different physical and chemical properties, is reported. By taking advantage of this property of the enzyme, a method of critical differential heat denaturation has been evolved as a method for the further purification of antitoxins.

The specific action is not limited to fibrinolysin, but appears to be a property of all the proteolytic enzymes provided they are used under the correct conditions. To be of use for this method the action of the enzymes must be so limited that no hydrolysis or digestion in its generally accepted sense takes place ; if this occurs differential denaturation fails, because obviously it cannot affect non-coagulable protein fragments.

Based on the methods outlined, a process for the large scale purification of antitoxins has been evolved, and will form the subject of other papers. As a point of interest it may be stated that by using these methods, antitoxin of such purity that all the protein present can be specifically precipitated by diphtheria toxin has been prepared experimentally.

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CRYSTALLINE PREPARATIONS OF TOMATO BUSHY STUNT VIRUS.

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IT has been shown that proteins, with characteristic properties and infective at high dilutions, can be isolated from plants infected separately with each of three strains of tobacco mosaic virus, two strains of potato virus " X ", and cucumber viruses 3 and 4 (Bawden and Pirie, 1937*a*, *b* ; 1938*a*). The proteins have not been found in healthy plants, but have been isolated from different species of infected plants. The particular protein isolated is determined solely by the infecting virus, is independent of the species of host plant, and has the stability typical of that virus. It is highly probable that the proteins are the viruses themselves—a view now becoming more widely accepted (Laidlaw, 1938)—but proof of this can be obtained only by a conclusive demonstration

that the protein preparations are pure. This, however, is impossible in practice, but experiments of a number of different kinds have failed to show any appreciable heterogeneity.

Although these viruses have different physical and chemical properties they are all nucleoproteins with similar analytical compositions, and they all have rod-shaped particles. Because of their shape they are readily orientated and the purified preparations have some properties characteristic of crystalline materials, but no true crystals, *i. e.* visible solids in which the particles are arranged with a three-dimensional regularity, have yet been produced *in vitro*. Concentrated solutions are liquid crystalline, dilute solutions show the phenomenon of anisotropy of flow, and birefringent jellies can be prepared. The solids formed by precipitating potato virus " X " with acids or salts are amorphous, while the precipitates of tobacco mosaic type viruses consist of needle-shaped paracrystals or micro-tactoids. These have been described as true crystals (Stanley, 1937 ; Wyckoff and Corey, 1936), but they are one of the forms of the liquid crystalline state, for the constituent particles are regularly arranged in two dimensions only (Bernal and Fankuchen, 1937).

We have now isolated from plants infected with Bushy stunt virus (Smith, 1935 ; Ainsworth, 1936 ; Bawden and Pirie, 1938*b*) a protein with the characteristic properties of this virus, and which has not been found in healthy plants. Like those previously isolated it is a nucleoprotein, but it differs from them in its analytical composition and in many other respects. Its particles are probably spherical, or at least not appreciably elongated, and the purified preparations, both liquid and solid, are isotropic. When precipitated with salts this virus forms true crystals, mainly rhombic dodecahedra ; these belong to the cubic system and, as would be expected, are isotropic. There is no conclusive evidence that our preparations of Bushy stunt virus are pure, but as they are fully crystalline, and show a high degree of homogeneity in different tests, a stronger claim can be made for their purity than for the liquid crystalline preparations of the other viruses, and in this paper the protein will be called virus.

METHODS.

The original source of the virus used in this work was an infected tomato plant supplied by Dr. Kenneth M. Smith. Bushy stunt virus is more difficult to transmit than the viruses we have previously investigated and the reactions of infected plants are more variable. It produces necrotic, local lesions on a number of species, including tobacco and *Nicotiana glutinosa*, but systemic infection has been obtained only in tomato and *Datura stramonium*. Most of our isolations have been made from tomatoes, but the same protein has been isolated from infected *Datura*.

The reasons for the variations in symptoms in tomato are not fully understood, but the reactions depend greatly upon the age and condition of growth of the inoculated plants. The first symptoms are local necrotic rings, and often no further effect is obtained. Systemic infection is first shown by a curling downwards and inwards of the petiole of the inoculated leaf, and by the check in growth of the main stem. Young seedlings then develop a severe

general bright yellow mottle with necroses, and are sometimes killed. Older plants react less severely, usually developing only scattered yellow blotches and necroses. Plants infected for a period of some weeks may produce numerous side shoots, giving the plants a characteristic bushy appearance from which the disease takes its name.

The yields of virus isolated have averaged about 50 mg. per litre of expressed sap, but they have varied widely, and are directly correlated with the severity of the symptoms shown by the plants. Sap from seedlings showing the severe general mottle may contain as much as 0·5 mg. of virus per c.c., while that from systemically infected plants showing only scattered blotches may contain less than 0·02 mg. per c.c.

Young plants are therefore used and cut down about three weeks after infection when they are showing the most definite symptoms, minced in a meat mincer and the sap expressed through bolting silk. The sap is heated to 60° C., cooled, and the bulky green coagulum produced is removed by a few minutes' centrifugation at 3000 r.p.m. The clear brown supernatant fluid is rather more than one-quarter saturated with ammonium sulphate by the addition of 200 gm. per litre. A small fawn-coloured precipitate separates, which readily sediments and packs tightly after a short period of centrifugation at 3000 r.p.m. The coloured supernatant fluid is discarded, and the precipitate is suspended in a volume of water equal to about one-tenth of the volume of original sap. The fluid is centrifuged until clear, and the preparation is further concentrated by a second precipitation with quarter-saturated ammonium sulphate solution. The virus-containing precipitate now dissolves in water to give a clear but darkly coloured solution, from which a small amount of insoluble material can be removed by centrifugation.

The type of precipitate of Bushy stunt virus produced by ammonium sulphate depends largely on the conditions of precipitation. Rapid precipitation in the presence of much salt gives amorphous material, while slow precipitation gives crystals. The virus has different solubilities in the two states, the amorphous material being considerably more soluble in dilute ammonium sulphate solution at 0° C. than at room temperature, whereas the crystals are not. By applying these two properties of the virus it can be freed completely from coloured contaminants, and obtained in a fully crystalline state with careful precipitations with ammonium sulphate. In this respect Bushy stunt virus differs sharply from tobacco mosaic virus and potato virus " X ", for they adsorb impurities much more readily, and it is usually impossible to obtain colourless, homogeneous preparations of them merely by repeated precipitations with acids and salts.

A saturated solution of ammonium sulphate is added slowly to the coloured virus preparations at room temperature with constant stirring until there is a trace of opalescence not dissipated by stirring. If kept at room temperature the opalescence soon increases and precipitation is complete in about an hour, but the material which separates is amorphous and darkly coloured, and better fractionation is obtained at 0° C. Unless too much ammonium sulphate has been added, the amorphous precipitate dissolves and the fluid becomes clear on cooling to 0° C., but after some time, the exact time depending upon the pH value and the virus content of the preparation, a crystalline deposit separates,

appearing first on the side walls and later as a precipitate at the bottom of the vessel. The process of crystallization at 0° C. is allowed to proceed for about 48 hours, when the fluid is centrifuged for a few minutes in a chilled centrifuge. The supernatant fluids are decanted, and as they warm up to room temperature a coloured amorphous precipitate separates, from which more virus can be recovered.

Bushy stunt virus differs greatly from the other viruses we have purified in that there is apparently no pH value at which it is insoluble in water or dilute salt solutions. At about pH 5, however, the crystals that separate from ammonium sulphate solutions dissolve only slowly in water. In contrast, precipitated amorphous material dissolves rapidly. The crystals are therefore washed by being suspended in about three times their volume of water and then centrifuged off again immediately. The rapid washing dissolves any amorphous pigmented materials, and these are removed in the supernatant fluid. The crystalline deposits are again suspended in water and left for an hour with frequent stirring; the crystals dissolve and a small amount of insoluble material can be removed by centrifugation.

Preparations of the virus at this stage are faintly opalescent but almost colourless. They will usually crystallize completely if the process described above is repeated. The initial turbidity at room temperature is produced by the addition of from one-sixth to one-fifth of a volume of saturated ammonium sulphate solution, and crystallization at 0° C. is complete in 2 days. The crystals are centrifuged off at or near 0° C., rapidly washed in water, and then dissolved in water. The solution is brought to about pH 3 by the addition of HCl and thoroughly dialysed against distilled water. Dialysis at pH 3 often gives a further fractionation not obtained at pH 7, for a colourless or fawn precipitate, consisting of a mixture of carbohydrate and denatured virus, separates and is centrifuged off when dialysis is complete.

The crystallization mother liquors and the water used for washing the crystals contain some virus. This can be recovered, after further concentrating the solutions by precipitation with ammonium sulphate, by repeating the process of slow crystallization at 0° C. Alternatively, it can be recovered by sedimenting the virus in a high-speed centrifuge or by incubating the preparations with pancreatin, which destroys contaminating proteins but not the virus, and then crystallizing the virus with ammonium sulphate. Incubation with pancreatin is the more convenient method, for Bushy stunt virus sediments more slowly in a high-speed centrifuge than viruses of the tobacco mosaic type. When a 1 per cent. solution of the virus is centrifuged for 2 hours at 14,000 r.p.m. in a rotor of 8 cm. radius only about one-quarter of the virus sediments. The pellets of sedimented virus are clear, and differ from those of tobacco mosaic virus and potato virus " X " in being isotropic. The material finally prepared as crystals has identical chemical, physical and serological properties regardless of the method used for purification.

The preparations can be crystallized completely so that microscopic examination of the ammonium sulphate precipitates shows only crystals in the form of rhombic dodecahedra. In order to obtain homogeneous preparations of this type it is essential to do the crystallization in dilute ammonium sulphate solution at 0° C., for preparations wholly crystalline in these

conditions may give amorphous precipitates if much ammonium sulphate is added at room temperature. It is noticeable that larger crystals are formed from slightly impure preparations than from those which have been repeatedly crystallized. This effect is illustrated in Fig. 1. The crystals were photographed on the wall of a vessel in which the same preparation was twice crystallized. The larger crystals were produced by the first crystallization and the smaller ones by the second. In addition to the rhombic dodecahedra, occasionally a few icositetrahedra are formed when slightly impure preparations are allowed to crystallize slowly, and at the top of the fluid there can usually be found crystals which appear to be flat hexagons. Such crystals have been isolated by Dr. F. M. L. Sheffield and inoculated to plants. They caused typical Bushy stunt symptoms, and it is therefore probable that they are merely malformed crystals of the same protein, produced by unequal crystal growth.

Preparations of Bushy stunt virus can also be crystallized with sodium sulphate and magnesium sulphate, but these offer no apparent advantage over ammonium sulphate. The crystal form is the same, and the precipitated material at room temperature is again largely amorphous and more soluble at 0° C. than at room temperature.

Tobacco mosaic virus and potato virus " X " form with clupein sulphate insoluble complexes having microscopic appearances similar to the precipitates of these viruses produced with acid and ammonium sulphate. The addition of clupein sulphate to salt-free solutions of Bushy stunt virus produces an amorphous precipitate, which resembles the ammonium sulphate precipitate in being more soluble at 0° C. than at room temperature. Also, after standing for some time at 0° C. a part of the preparation turns into rather misshapen dodecahedra. The clupein complexes of all three viruses have similar properties ; they contain 5 per cent. or less of clupein sulphate and dissolve in salt solutions more concentrated than M/10, but the minimum value necessary for solution depends on the salt used, the pH value, and the amount of clupein sulphate present.

Nothing whatever can be isolated from healthy tomato plants by the purification method described. This is largely the result of two of the processes used ; heating the sap to 60° C. denatures most of the normal plant proteins precipitated by one-quarter saturated ammonium sulphate solution, and the residual protein is no more soluble in ammonium sulphate solution at 0° C. than at room temperature. The behaviour of some of the constituents of normal plant sap is described in the accompanying note.

The solid content of the purified virus preparations is determined by freezing a sample and drying it *in vacuo* over P_2O_5 while frozen. The dried material is highly hygroscopic, and rapidly adsorbs from 5 to 8 per cent. of water on exposure to the air. The dried preparations differ from those of tobacco mosaic virus for they are completely insoluble in water or in dilute salt solutions. Solutions of the virus have been dried at a number of different pH values, in the absence of salts and in various buffer solutions, frozen and unfrozen, but all were insoluble and quite inactive. Freezing and thawing was found to have no effect on the viruses with which we have previously worked, but it denatures Bushy stunt virus. Precipitates develop in solutions frozen and thawed and the virus is inactivated.

RESULTS.

Serological Reactions and Infectivity.

Bushy stunt virus is antigenic, and sera giving specific precipitates at dilutions of over 1/1000 were prepared by giving rabbits a single intravenous injection of 2 mg. of purified virus. The serological titres of the virus preparations have largely been used to determine activity, for they are much more constant than infectivity tests, which vary widely with individual plants. The titres were determined by adding 1 c.c. of antiserum at a constant dilution to a series of tubes each holding 1 c.c. of solution containing a known weight of antigen, and they are expressed as the smallest weight in grammes per c.c. of antigen solution to give a precipitate visible to the naked eye. The tubes were kept for 12 hours with the fluid columns half immersed in a water-bath at 50° C., and the readings were made after a further 12 hours at room temperature.

The serological titres of the crystalline Bushy stunt virus preparations have been consistently lower than those of the liquid crystalline preparations of tobacco mosaic virus and potato virus " X ". This difference probably has its origin in the different shapes of the viruses. The precipitates of tobacco mosaic virus and virus " X " with their antisera are of the same type, but differ greatly from those of Bushy stunt virus. They form rapidly, are flocculent, have an open structure, and after settling take up a large volume, *i. e.* they closely resemble the precipitates obtained with bacterial flagellar (" H ") antigens. On the other hand, the precipitates of Bushy stunt virus with its antiserum are of the type obtained with bacterial somatic (" O ") antigens. They form more slowly, are granular, and settle out into compact masses at the bottom of the tubes. The differences are illustrated in Fig. 2. The central tube contains saline. The three left-hand tubes contain 1 c.c. of Bushy stunt antiserum at 1/50 and 0·1, 0·05 and 0·025 mg. of Bushy stunt virus in 1 c.c. respectively. The three right-hand tubes contain 1 c.c. of tobacco mosaic virus antiserum at 1/50 and 0·1, 0·05 and 0·025 mg. of tobacco mosaic virus in 1 c.c respectively. After adding the antisera to the virus solutions, the tubes were immediately placed in the water-bath at 50° C. A precipitate was at once obvious in the most concentrated solution of tobacco mosaic virus, and within two minutes there was a bulky precipitate in this and precipitation was also apparent in the more dilute solutions. The first signs of precipitation were not seen in the most concentrated solution of Bushy stunt virus until the tubes had been in the bath for 6 minutes, and it was 30 minutes before the most dilute solution precipitated. After 12 hours in the water-bath the tubes were left at room temperature, and they were photographed after standing undisturbed for a further 12 hours.

The particles of tobacco mosaic virus and of potato virus " X " can be readily orientated by streaming and this, and other allied phenomena, show that they are greatly elongated. Structurally, therefore, the particles of these viruses will resemble flagella. It is to be expected that when these rods flocculate and come together they will become entangled and support one another, giving a fluffy open structure. The particles of Bushy stunt virus cannot be orientated by streaming, and there is no evidence to suggest that

they are not spherical. Spheres, coming together at random, will pack more tightly than rods, and a precipitate composed of spheres will probably be more compact than one made up of rods. Thus, for a given weight of antiserum precipitate, it is to be expected that either potato virus " X " or tobacco mosaic virus will produce a more voluminous and easily seen precipitate than Bushy stunt virus, and, if the viruses precipitate similar amounts of serum protein at the antigen dilution end-point, it is to be expected that the rod-shaped viruses will give visible precipitates at higher end-points than Bushy stunt virus.

There are no measurements of the ratio of antigen to antibody in these virus-antiserum precipitates. However, Boyd and Hooker (1934 ; 1936) have pointed out that the amount of antibody combining with a given amount of antigen corresponds closely with the amount necessary to form a monomolecular layer of antibody particles over the surface of the antigen particles, and with the existing knowledge on the sizes of the virus particles this ratio can be calculated roughly. If the tobacco mosaic virus particles are long prisms the ends can be neglected and it is unnecessary to know their length, for the ratio of antigen to antibody will be approximately the ratio of the cross-sectional area of the virus to the area of a ring of antibody particles on the surface of the virus particle. The cross-sectional area is 20,100 sq. A (Bawden and others, 1936) and, within the degree of accuracy needed for this argument, we may regard the rod as a cylinder of about 8 mμ. radius, although it is highly probable that the rods do not have a strictly circular section. Kabat and Pedersen (1938) give the molecular weight of rabbit pneumococcus antibody as 157,000, and presumably the antibodies to the viruses will have a similar value. Such a particle, if spherical, would have a radius of about 3·7 mμ. As a first approximation, therefore, the ratio of virus to antibody in the serum precipitates of tobacco mosaic virus should be the ratio of the area of a circle of radius 8 mμ. to the area of the ring between circles of 8 mμ. and 11·7 mμ. radius, *i. e.* 1 : 1·1.

As Bushy stunt virus appears to have a spherical particle, its molecular weight can be legitimately calculated from its sedimentation constant. McFarlane and Kekwick (1938) find the sedimentation constant to be $S_{20} = 146 \times 10^{-13}$ and the density to be 1·353, and give the molecular weight as 8,800,000 and the radius as 13·7 mμ. With this virus, therefore, the ratio of antigen to antibody in the precipitates will be approximately the ratio of a volume of a sphere of 13·7 mμ. radius to the volume of a shell of 13·7 mμ. internal radius and 17·4 mμ. external radius, *i. e.* 1 : 1·0. The only large particles of size comparable with the viruses for which such ratios have been measured are the hæmocyanins (Boyd and Hooker, 1936), and the values of from 0·6 to 1·4 agree sufficiently well with our calculated ratios to suggest that they are of the right order. It seems reasonable to assume, therefore, that tobacco mosaic virus and Bushy stunt virus precipitate comparable amounts of antibody, and that this quantity is approximately equal to the weight of virus in the system at optimal proportions, and not greatly in excess of it in the region of antibody excess. When particles of the size of these viruses give precipitation end-points of 10^6, the precipitate in 2 c.c. final volume of fluid will be made up of 0·001 mg. of virus and not much more than 0·001 mg.

of antibody, *i. e.* the precipitate has a weight of about 0·002 mg. Other more or less spherical antigens whose serological titres have been determined are mainly those with molecular weights of under 100,000. These will have a greater surface to weight ratio than the larger antigens, and the antibody to antigen ratio would be expected to be correspondingly greater. The figures collected by Marrack (1938) range from 10 to 21 in the region of antibody excess, and with such antigens a titre of 10^6 corresponds to from 0·01 mg. to 0·02 mg. of precipitate. It is to be expected, therefore, that large spherical antigens, such as Bushy stunt virus, will give lower serological titres than smaller spherical antigens, or than greatly elongated antigens giving precipitates of an especially easily visible type.

The serological titres of Bushy stunt virus preparations are more dependent on the concentration of antiserum used than are those of tobacco mosaic virus, for inhibition of precipitation by excess of antibodies is more definite. The effect of varying the concentration of antiserum on the precipitation of a Bushy stunt virus preparation is shown in Table I. Because of this effect the titres were determined with antiserum diluted at least 1/200 with 0·85 per cent. NaCl solution.

TABLE I.—*Effect of Antiserum Concentration on Precipitation of Bushy Stunt Virus.*

Antiserum.	Time.	Antigen in mg. per c.c.								
		0·2.	0·1.	0·02.	0·01.	0·005.	0·0025.	0·002.	0·0016.	0·0012
1/5	2 min.	++	−	−	−	−	−	−	−	−
	30 „	+++	++	−	−	−	−	−	−	−
	90 „	++++	+++	±	−	−	−	−	−	−
	24 hr.	++++	++++	+++	+	±	−	−	−	−
1/50	2 min.	++	−	−	−	−	−	−	−	−
	30 „	++	+++	±	−	−	−	−	−	−
	90 „	++++	+++	++	+	−	−	−	−	−
	24 hr.	++++	++++	+++	++	+	+	±	−	−
1/500	30 min.	−	−	±	−	−	−	−	−	−
	90 „	−	−	++	+	±	−	−	−	−
	24 hr.	−	−	+++	++	+±	+	+	+	±

1 c.c. of antiserum at the stated concentration added to 1 c.c. of antigen solution containing given weight of antigen. Tubes were immediately placed in the water-bath at 50° C.
+ signs indicate the degree of precipitation.

Infectivity tests were made by the local lesion method, *Nicotiana glutinosa* being used as the host. The lesions resemble those produced by tobacco mosaic virus, but if equal weights of the two viruses are inoculated to opposite halves of the same leaves the tobacco mosaic virus produces many more lesions. The results of precipitation end-point determinations and infectivity tests on five different crystalline preparations of Bushy stunt virus are shown in Table II. The precipitation end-points of a large number of preparations have all been similar, with antiserum at a dilution of 1/200, varying only between 1 : 600,000 and 1 : 800,000. With antiserum at 1/800 titres of

TABLE II.—*Activity of Crystalline Preparations of Bushy Stunt Virus.*

Serological titre.	Infectivity on *N. glutinosa*. Average number of lesions per leaf at various dilutions.					
	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.
1 : 800,000	84	31	11	1	0·4	0
1 : 800,000	..	37	20	2·5	0·5	0
1 : 600,000	48	28	11	1	0	0
1 : 600,000	81	20	7	1·5	0·5	0
1 : 800,000	74	53	11	4	0·4	0·2

Serological titre is expressed as the smallest amount, in gm. per c.c., of antigen giving a visible precipitate with 1 c.c. of antiserum at 1 in 200. Dilutions in infectivity tests given as gm. per c.c., and 1 c.c. is rubbed over from 5 to 6 leaves.

$1/10^6$ have been obtained. The activity of a fully crystalline preparation is unaffected by recrystallizations or by repeated sedimentations in a high-speed centrifuge, but the activity of partially purified preparations is increased by these treatments. Further evidence that the virus activity is in the crystals was obtained by inoculating isolated large crystals, after washing in one-quarter saturated ammonium sulphate solution and dissolving in water, to *N. glutinosa* and tomato, when typical symptoms were obtained.

Analyses and Isolation of Nucleic Acid.

Analytical figures have been determined on a number of fully dried preparations and have fallen in the following ranges :

Carbon	47 to 50 per cent.	Hydrogen	7·2 to 8·2 per cent.
Nitrogen	15·8 ,, 16·4 ,,	Sulphur	0·4 ,, 0·8 ,,
Phosphorus	1·3 ,, 1·5 ,,	Carbohydrate	5 ,, 6 ,,
Ash	1·7 ,, 5 ,,		

Phosphorus was determined by the method of Kuttner and Lichenstein (1932). The carbohydrate estimations were made by an orcin method of Pirie (1936) with glucose as a standard and the figures given are referred to glucose. As the sugar of the virus is probably ribose, to refer the figures to ribose they should be multiplied by 0·84. The other constituents were determined by the usual Pregl methods.

Unless the virus has been carefully prepared the carbohydrate content may be 8 per cent. The extra 2 to 3 per cent. of carbohydrate can be removed, without inactivating the virus, either by recrystallizing and thoroughly washing the crystals with distilled water, or by repeatedly sedimenting the virus in a high-speed centrifuge. This carbohydrate differs from that found contaminating partially purified preparations of potato virus " X " (Bawden and Pirie, 1938*a*), for that was insoluble in water and always associated with coloured material. The presence of the extraneous carbohydrate in preparations of Bushy stunt virus can be demonstrated by extracting from 5 to 10 mg.

of the dried virus with 1 c.c. of $N/20$ ammonium hydroxide for a few hours at room temperature. This treatment extracts nothing from preparations with a carbohydrate content of from 5 to 6 per cent., for the protein is insoluble and the nucleic acid is not extracted unless the mixture is heated, but any contaminating carbohydrate dissolves and can be estimated in the usual way. Alternatively, it can be isolated from preparations inactivated by freezing and thawing; the insoluble virus can be removed from the fluid by centrifugation, but the excess carbohydrate remains in the clear supernatant fluid. This method is the most convenient for the routine examination of the virus preparations, and detects from 0·5 to 1·0 per cent. of contaminating carbohydrate.

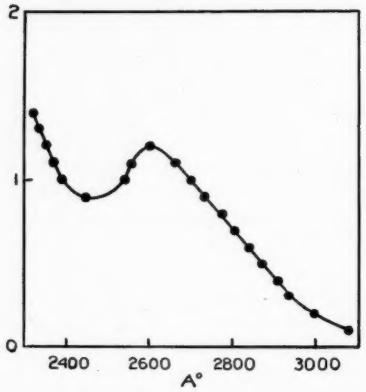


FIG. 4.—Ultra-violet absorption curve of a solution of Bushy stunt virus.

The carbon, hydrogen and nitrogen figures are very similar to those obtained with tobacco mosaic virus and potato virus "X", and to those of proteins in general, but the phosphorus and carbohydrate figures are more than twice as great, suggesting that Bushy stunt virus has a higher nucleic acid content. This suggestion is reinforced by a study of the ultra-violet absorption spectra of the viruses. The tobacco mosaic group of viruses give general absorption beginning at about 2500 A and also a definite absorption maximum at 2600 A (Bawden and Pirie, 1937*a*), the region generally associated with absorption by nucleic acid. Solutions of Bushy stunt virus absorb ultra-violet light even more strongly at 2600 A; with tobacco mosaic virus $\log_{\cdot 10} I_0/I$ is 1·2 at 2600 A with a 2 cm. column of a 0·02 per cent. solution, whereas, under the same conditions, this value is given by a 0·012 per cent. solution of Bushy stunt virus. Fig. 4 is the absorption curve of a solution of Bushy stunt virus.

In Bushy stunt virus the nucleic acid is more firmly bound than in the plant viruses we have previously studied. A few minutes' heating of salt-free solutions at 90° C. inactivates the virus, but even when boiled such solutions

remain clear. On the addition of a little salt to the boiled solutions a protein coagulum separates, but carries with it all the nucleic acid. Extraction of the virus inactivated by either heating or drying with boiling $N/80$ ammonia solution dissolves most of the nucleic acid and the residual protein has a low phosphorus content. The addition of acid causes the fluid to become opalescent, and in a few hours a precipitate forms with the typical resinous character of nucleic acid. The precipitate is ground up with a little water and washed by centrifugation, and the residue dissolves completely when sufficient alkali is added to make the preparation neutral. After reprecipitation with acid, the sodium nucleate is dried in the frozen state and gives a colourless, easily soluble, feathery mass containing 6 to 7 per cent. of phosphorus and from 27 to 31 per cent. of carbohydrate. It gives neither a Schiff reaction for desoxypentose nor Dische's reaction with diphenylamine (1930) after hydrolysis with acid, but it gives as much colour as the same weight of yeast nucleic acid by McCance's (1926) method of pentose estimation. There is not sufficient of the nucleic acid available for a rigorous identification of the carbohydrate, but it seems reasonable to conclude that the nucleic acid is of the ribose type similar to that in yeast and tobacco mosaic virus (Bawden and Pirie, 1937*a*). The value of the specific rotation, $[\phi]_D^{20^\circ} = 58^\circ \pm 4^\circ$, supports this conclusion.

Material, apparently identical with that obtained by extracting the virus with boiling alkali, can be prepared by incubating the coagulum produced by heating the virus in the presence of a little salt or acid with trypsin free from nuclease.* After the coagulum has been digested the nucleic acid can be precipitated with acid. Bushy stunt virus is destroyed by warm acetic acid and by wetting and spreading agents such as sodium dodecyl sulphate (Bawden and Pirie, 1938*a*). A phosphorus-free protein can be precipitated with ammonium sulphate at neutrality from preparations inactivated by these agents, and from the supernatant fluids the nucleic acid can be isolated by precipitation with acid.

The amount of nucleic acid isolated is from 15 to 20 per cent. of the weight of virus taken. In view of the practical difficulties in isolating substances of this kind on a small scale, the yield accounts satisfactorily for so much of the phosphorus and carbohydrate of the virus preparations that there is no reason to suspect the presence of any phosphorus or sugar containing components other than the nucleic acid.

The ash content of preparations recrystallized several times with ammonium sulphate and dialysed for many days against distilled water at from pH 3 to pH 5 does not usually fall below 3 per cent. Some of this tightly bound ash can be removed by electrodialysis against distilled water, but an ash-free preparation has not yet been obtained.

Effect of Acids and Alkalis.

Although so sensitive to dehydration, Bushy stunt virus is less affected by changes of pH than the other viruses we have studied. It does not precipitate, and undergoes no apparent change during exposure of some hours at room

* The trypsin preparation was kindly supplied by Dr. J. H. Northrop.

temperature to pH values of between 1·5 and 9·5. At pH 1 the virus solutions rapidly lose their original faint opalescence and become water-clear. Nothing can be sedimented from such solutions by high-speed centrifugation, but on neutralization the protein precipitates and the virus is inactivated. Exposure to pH 10 or higher for a few hours at room temperature destroys the infectivity. No precipitate develops in these solutions on neutralization unless they are kept for about 48 hours, when a part of the material separates. If this is removed by centrifugation the remaining solution is found to be still capable of reacting with antiserum, although it is non-infective. The effect of exposure to different pH values is shown in Table III.

TABLE III.—*Effect of Acid and Alkali on Bushy Stunt Virus.*

pH value.	Serological titre.	Infectivity at 10^{-4}. Average number of lesions per leaf.
1	..	0
1·5	1 : 600,000	41
7	1 : 600,000	38
9·5	1 : 600,000	51
11	1 : 200,000	0

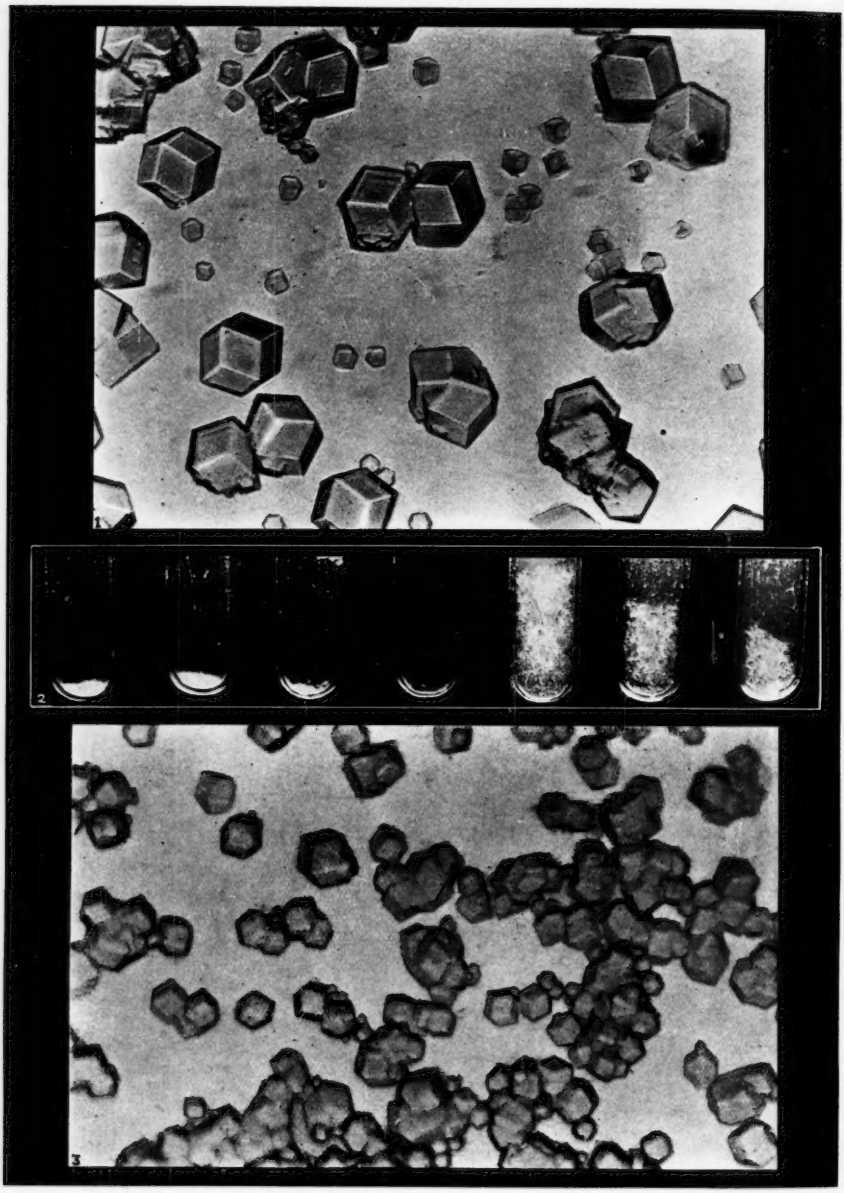
Mixtures left for 2 hours at 18° C. at the pH stated.

Inactivation with Nitrous Acid and Ultra-violet Light.

Tobacco mosaic virus and potato virus "X" can be inactivated with nitrous acid and ultra-violet light without being denatured, and without losing their serological properties or their characteristic optical properties (Bawden and Pirie, 1937*a*; 1938*a*). Bushy stunt virus is also inactivated by these treatments without appreciably modifying its serological or physical properties.

Provided the solution is kept cold, no denaturation takes place when solutions are irradiated with ultra-violet light under a quartz plate. A 1·5 per cent. solution irradiated for 40 minutes at 8 cm. from a 2 amp. mercury vapour lamp was found to be non-infective, but gave a precipitation end-point of 1 : 600,000. On the addition of ammonium sulphate it crystallized well. The crystals were again dodecahedra, but showed a greater tendency to form crystal aggregates than the fully active virus (Fig. 3). The inactive preparations can be recrystallized, and after dialysis dissolve in the same manner as the active virus.

Exposure for 10 hours at 0° C. and at pH 3·4 in the presence of 1·2 per cent. sodium nitrite destroyed the infectivity of a 0·68 per cent. solution of the virus without affecting its precipitation end-point with antiserum. The protein precipitated in the usual way on the addition of ammonium sulphate, and when recrystallized formed dodecahedra showing all the properties associated with the active virus. Treatment with formaldehyde and hydrogen peroxide also inactivates Bushy stunt preparations without destroying their crystallinity or serological reactions.



Bawden and Pirie.

SUMMARY.

The isolation of a protein, probably the virus itself, is described from plants infected with tomato Bushy stunt virus. This protein not only differs from the normal plant proteins, but it also differs more from the other purified plant viruses than these differ from one another. It is fully crystalline instead of liquid crystalline. It has a higher nucleic acid content than tobacco mosaic virus or potato virus " X ", and is more stable towards pH changes, but less stable towards dehydrating agents. Its particles are not elongated, and liquid and solid preparations are isotropic. 1 c.c. of solution containing 10^{-7} gm. produces infections when rubbed on to *N. glutinosa*, and 1 c.c. containing 10^{-6} gm. gives a specific precipitate with antiserum. Precipitates of the rod-shaped viruses with their antisera resemble those obtained with bacterial flagellar (" H ") antigens, but those of Bushy stunt virus resemble those with somatic (" O ") antigens. When irradiated with ultra-violet light or treated with nitrous acid the virus loses its infectivity, but it can still be crystallized and still retains its serological activity.

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FIG. 1.—Dodecahedral crystals of Bushy stunt virus. The larger crystals were produced by a first crystallization with ammonium sulphate and the smaller by a second. × 250.

FIG. 2.—Precipitates of Bushy stunt virus and tobacco mosaic virus with their homologous antisera. Central tube saline control. Three left-hand tubes, 0·1, 0·05 and 0·025 mg. of Bushy stunt virus; three right-hand tubes, same weights of tobacco mosaic virus. Note compact precipitate of Bushy stunt and bulky fluffy precipitate of tobacco mosaic virus.

FIG. 3.—Crystals of Bushy stunt virus inactivated by irradiation with ultra-violet light. Material is still serologically active. × 300.

A NOTE ON SOME PROTEIN CONSTITUENTS OF NORMAL TOBACCO AND TOMATO LEAVES.

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In the course of work on the isolation of a number of viruses from infected tobacco (var. White Burley) and tomato (var. Kondine Red) plants we have gained some information on the behaviour of the proteins of normal plants. Some heat-stable proteins have already been described (Bawden and Pirie, 1937), and this note deals only with unstable proteins, some with molecular weights of the same order as the viruses, which must be removed during the isolation of the viruses. The proteins characteristic of the viruses already isolated differ so definitely from these normal proteins that they cannot easily be confused with them, and their purification presents few difficulties. Others, however, especially the relatively unstable insect-transmitted viruses such as potato virus "Y", cucumber virus 1, and *Hyoscyamus* virus 3, have properties more nearly resembling those of the normal plant proteins, and their isolation is therefore more difficult.

Different samples of sap from healthy plants differ so greatly in their protein contents and in the properties of the protein that it is impossible to make confident statements, but some material with the properties to be described is always found in both healthy and virus-infected plants. Also, the proteins are so readily changed irreversibly that it would be unwise to assume that precipitation with salts or sedimentation by high-speed centrifugation does not alter them, and we have no evidence that they have the same properties in the cell or in untouched sap.

The pH of expressed tobacco or tomato sap is usually just below 6 and is a little lower with sap from older plants. It can be cleared by centrifuging at 3500 r.p.m., but it clears more rapidly if disodium hydrogen phosphate is added. The addition of NaOH to bring the pH to 8 has no apparent effect, but the addition of the phosphate causes a precipitate of calcium compounds and proteins to separate, amounting to about 3 gm. from a litre of sap. The addition of ammonium sulphate to the clarified sap causes a bulky precipitate at one-quarter saturation. The precipitate dissolves readily in water at neutrality to give a darkly coloured viscous solution. These solutions become opalescent when one-seventh saturated with ammonium sulphate, and after a

few hours a precipitate separates, and can be removed by centrifugation. The addition of a little more ammonium sulphate to the supernatant causes a further precipitate, and a series of fractions is obtained differing in the amount of salt required to precipitate them, but not differing appreciably in any of their other properties studied. All the fractions dissolve readily in water at pH 7 and are precipitated when the pH is lowered to about 5. The precise value depends on the acid used; in general phosphoric acid precipitates the proteins more easily than other weak acids such as phthalic and acetic. The precipitation is irreversible, for 90 per cent. of the material remains insoluble at pH 7, although it can still be dissolved by the addition of excess acid.

It seems that the molecules of some of these proteins are large, for they are readily sedimented from neutral solution by high-speed centrifugation. From one-tenth to one-third of the protein usually sediments when a 0·5 per cent. solution is centrifuged for 2 hours at 12,500 r.p.m. in a rotor of 8 cm. radius. The sedimented material gives brown jellies with a similar appearance to those obtained by centrifuging crude virus preparations. The jellies are isotropic, and are easily dissolved or dispersed in water. The protein can again be sedimented and the process repeated, until the protein from a redissolved pellet sediments at the same rate as that in the supernatant poured off from it. It seems probable that these apparently homogeneous proteins with high molecular weights are normal constituents of a large number of plant species, and their existence throws some doubt on the purity of virus preparations made solely by the high-speed centrifugation of infective sap. We have found them in both healthy and virus-infected tobacco and tomato plants, and the materials centrifuged from cucumbers by Price and Wyckoff (1938) and from peas and beans by Loring, Osborn and Wyckoff (1938) are presumably proteins of a similar nature. Wyckoff, Biscoe and Stanley (1937) found no such proteins in tobacco, and they state that healthy tobacco sap contains no molecules with a weight greater than 30,000, but it is possible their treatment of the leaves before expressing the sap may account for this.

At room temperature these proteins denature and precipitate in a few days, but if kept cold and neutral they are reasonably stable. They are sensitive to freezing, which converts them irreversibly into an insoluble material. As the protein is not apparently affected by exposure to high salt concentrations for short periods, the freezing must either be done at very low temperatures or in the absence of salts, for otherwise much of the protein will be concentrated with the salt in unfrozen pockets of fluid. This effect may explain the somewhat variable results obtained when leaves are frozen before being minced for the extraction of sap. As these normal plant proteins and Bushy stunt virus (Bawden and Pirie, 1938) are denatured by freezing, it seems that freezing the tissues may be a dangerous preliminary to work on the labile protein constituents of plants. On the other hand, in isolating such viruses as tobacco mosaic virus and potato virus " X " which are apparently unaffected by freezing and thawing the treatment may provide a useful fractionation.

These normal plant proteins are denatured by a few minutes' heating at 55° C., and are readily destroyed by incubation with commercial trypsin preparations. In the presence of salts they are also sensitive to alcohol. The

addition of alcohol to salt-free solutions of the proteins causes an opalescence, but no precipitate separates until several volumes are added. In the presence of salts, especially of calcium salts, a precipitate separates at from 25 to 35 per cent. alcohol, and the precipitated material is insoluble in water.

Except for their high molecular weights these normal plant proteins have little in common with the plant viruses that have yet been isolated. They contain from 14 to 16 per cent. of nitrogen but less than 0·02 per cent. of phosphorus and, if repeatedly sedimented by high-speed centrifugation, less than 1 per cent. of carbohydrate, whereas all the viruses we have obtained in crystalline or liquid crystalline form have been nucleoproteins. Best (1937) and Loring (1938) have confirmed the fact that tobacco mosaic virus is a nucleoprotein. The proteins have no serological relationship with any of the viruses we have studied. They also differ from the viruses in being relatively poor producers of precipitating antibodies when injected intravenously into rabbits. No evidence has yet been obtained suggesting a possible mechanism for the multiplication of viruses, but the suggestion that they are autocatalysts produced from virus-precursors is frequently made. We feel that it would be extremely premature to look upon these normal plant proteins as in any sense virus precursors merely because of a similarity in molecular weight, especially as they differ so definitely from the viruses in their chemical, physical and serological properties. Tobacco mosaic virus has been added to concentrated solutions of these normal plant proteins, but so far from there being any evidence of virus increase, the dilution of the virus with the protein caused a greater loss in infectivity than a similar dilution in water or buffer solutions. Muller (1922) suggested that viruses might be "free genes", and since it has been found that certain viruses are nucleoproteins, the suggestion that viruses might be portions of nuclear material or the naked nuclei of hypothetical small bacteria has frequently been made. However, in this connection it should be noted that the nucleic acids characteristic of the nuclei of plant and animal cells contain desoxy pentose, whereas all the plant viruses that have been isolated have contained a nucleic acid of the ribose type.

In describing the isolation of tobacco mosaic virus (Bawden and Pirie, 1937) we commented on the larger amount of protein in clarified sap from infected plants than in that from healthy plants. The sap was usually prepared from frozen leaves and was often acidified slightly to facilitate clearing. It had therefore lost at least a part of the normal proteins described in this paper. Even when sap from unfrozen leaves is used and clarified by centrifuging there is a real difference, for the yield of unstable proteins in normal or infective sap does not exceed 2 gm. per litre, and this amount of virus can be isolated from a litre of infective sap. The soluble protein in the sap will of course represent only a portion of the total protein of the leaf, and Martin, Balls and McKinney (1938) have shown that there is no change in the total protein content of infected leaves at all comparable with the change in the content of soluble protein. This suggests that infection causes a change in the ratio of soluble to insoluble protein, and it is possible that a detailed study of the normal insoluble nucleoproteins of the cell might give some information on the mechanism whereby the abnormal nucleoproteins are formed in the infected plants.

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THE INDOPHENOL-REDUCING CAPACITY OF GUINEA-PIG LEUCOCYTES.

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IMPRESSED by the unusually high ascorbic acid content of the whole blood in patients suffering from leukæmia, as determined by indophenol titration, Stephens and Hawley (1936) followed up this observation by investigating the partition of ascorbic acid in the blood of a number of normal subjects and of patients suffering from a variety of diseases. Samples of blood from thirty individuals were examined in this connection. They found that, although the values varied widely, the ascorbic acid content of the leucocytes was consistently and significantly higher than that of the other fractions of the blood examined. The high titration values observed in patients with leukæmia were, therefore, ascribed to the preponderance of white cells in the blood.

It is mostly assumed that the rapid reduction of indophenol in acid solution by extracts of animal tissues is due entirely to the presence of ascorbic acid. This assumption is often but not invariably justified. Thus Johnson (1936) found that whilst in the humor of the eyes of the horse, ox, sheep, pig and guinea-pig the capacity for reducing indophenol was entirely due to ascorbic acid, the eye lens contained a substance or substances, other than ascorbic acid, which reduced indophenol. We, therefore, performed a series of experiments on guinea-pigs with the object of ascertaining whether in the case of leucocytes the reduction of indophenol was due entirely to ascorbic acid, and a few typical experiments will be described.

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TECHNIQUE.

The leucocytes were obtained by injecting guinea-pigs intraperitoneally with 20 ml. of sterile broth. After 16–24 hours the animals were killed by chloroform, and the exudate was removed from the peritoneal cavity by means of a capillary pipette into an isotonic citrate solution. A microscopic examination of the suspension revealed that it was almost entirely free from erythrocytes. This suspension was then centrifuged for 10 minutes and a firm deposit of leucocytes was thus obtained. After pouring off the supernatant fluid and removing the last traces by absorption with filter-paper, the deposit was ground with fine acid-extracted silver sand under 5 ml. of 5 per cent. trichloroacetic acid. The residue was then separated by centrifugation, adjusted to pH 3, and an aliquot portion of the extract titrated with 1·14 $N/1000$ indophenol from a microburette. When the yield of leucocytes permitted, another portion of the trichloroacetic acid extract was saturated and kept in contact with hydrogen sulphide overnight, and after completely displacing the hydrogen sulphide by a stream of nitrogen the solution was titrated with indophenol as before. This latter treatment was carried out in order to ascertain whether any reversibly oxidized ascorbic acid was present.

RESULTS.

The aim of these experiments was not only to assess the rapid indophenol-reducing capacity of the leucocytes of the guinea-pigs existing on a mixed diet containing a liberal supply of ascorbic acid, but also to determine this reducing capacity of the white cells of animals which had existed for varying periods on a scorbutic diet. In the former case all the tissues of the animal are " saturated " with ascorbic acid, *i. e.* their vitamin C content had reached a value which cannot be raised by further oral administration of ascorbic acid. When " saturated " guinea-pigs are, however, deprived of the vitamin, this store in the tissues disappears within a few days. In the lens of the eye, in which, as already mentioned, only part of the rapid indophenol reduction is due to ascorbic acid, some of this reducing capacity persists even after all the other tissues have completely lost this capacity. The substance or substances which thus persist in the eye lens of guinea-pigs subsisting on a scorbutic diet fail to show the selective absorption in the ultra-violet region of the spectrum characteristic of ascorbic acid.

The leucocytes of guinea-pigs kept on a scorbutic diet for 8, 15 and 21 days were, therefore, examined. In addition the indophenol-reducing capacity of the white cells from animals on a mixed diet and on a scorbutic diet which have received an intra-muscular injection of 50 mg. of ascorbic acid 24 hours before the collection of the leucocytes was determined. The object of this experiment was to ascertain whether the circulation in the blood-stream of large quantities of ascorbic acid, far higher than those that could be attained by oral consumption of the vitamin, would significantly raise the content of the white cells. The effect of limiting the consumption of a mixed diet so as to arrest growth of young guinea-pigs, but offering a very adequate supply of vitamin C in the form of cabbage and extra ascorbic acid, was also studied in this connection.

TABLE I.—*Indophenol-reducing Capacity of Leucocytes from Guinea-pigs on Mixed and Scorbutic Diets.*

Diet.	Number of guinea-pigs used.	Average weight of guinea-pigs. (g.)	Intramuscular injection of ascorbic acid. (mg.)	Weight of leucocytes. (g.)	Indophenol-reducing capacity calculated as ascorbic acid. (mg./100g.)	Indophenol-reducing capacity calculated as ascorbic acid after H_2S. (mg./100g.)
Mixed, cabbage *ad lib.*	3	522	None	0·602	6·9	7·0
„ „ „	4	292	50	0·524	9·9	8·7
Mixed, including cabbage, quantitatively restricted + 10 mg. ascorbic acid per day (21 days)	4	296	None	0·523	8·6	Not tested
Scorbutic (8 days)	4	296	„	0·473	2·6	„
„ (15 „)	5	310	„	0·824	3·3	3·3
„ (15 „)	4	332	50	0·902	4·9	4·7
„ (21 „)	6	281	None	0·263	1·9	Not tested

It will be seen from the table that in the case of the " saturated " guinea-pigs on a mixed diet the indophenol-reducing capacity was equivalent to 6·9 mg. of ascorbic acid per 100 g. of leucocytes, and that this value could only be raised to 9·9 mg./100 g. by introducing high quantities, *i. e.* 50 mg. of ascorbic acid into the blood-stream. With the quantitatively restricted diet and an adequate supply of vitamin C a similar figure of 8·6 mg./100 g. was obtained. On the other hand, after the animals had been depleted of their vitamin C supply by restricting them to a scorbutic diet for 8, 15 and 21 days, the reducing value fell to 2·6, 3·3 and 1·9 mg. per 100 g. of leucocytes respectively. The injection of 50 mg. of ascorbic acid 24 hours before analysis raised it only to 4·9 mg./100 g. Since dehydroascorbic acid is reduced in the animal organism, the absence of the reversibly oxidized form of ascorbic acid from the cells was to be expected.

The fact that the tissues of the guinea-pig part with *all* the stored ascorbic acid when the animal is deprived of vitamin C strongly suggests that in the case of the leucocytes, as in that of the eye lens, the residual substance which reduces indophenol rapidly, and which persists even after depriving the guinea-pigs of vitamin C for 21 days when declared symptoms of scurvy are present, is not ascorbic acid. Attempts were made to establish its identity spectrophotometrically, but, owing to the presence of interfering substances in the aqueous-alcoholic extract and to the difficulty of obtaining sufficient material, they had to be abandoned.

An attempt was also made to ascertain whether the residual indophenol-reducing capacity was due to the presence of high concentrations of sulphydryl compounds in the trichloroacetic acid extracts of cells. Two experiments were performed in which guinea-pigs weighing 500–600 g. were employed. In the first experiment 1·122 g. of leucocytes were obtained from four animals, and in the second three animals yielded 1·074 g. These leucocytes after being washed and centrifuged with isotonic citrate three times were extracted with trichloroacetic acid as described above, and to 4 ml. of the extract, 1 ml. of each of a solution of saturated ammonium sulphate, 25 per cent. ammonia and 1 per cent. sodium nitroprusside was added. A fugitive coloration characteristic of sulphydryl compounds was obtained. The tubes were then quickly matched against standards containing crystalline glutathione in 2·5 per cent. trichloroacetic acid. The trichloroacetic acid extracts of the leucocytes were thus found to show a coloration of about the same intensity as 7·5 mg./100 ml. of a glutathione solution. A trichloroacetic acid solution of glutathione of this strength was found, however, not to reduce indophenol at a speed which could simulate the presence of ascorbic acid. Whether the substance giving the nitroprusside reaction is identical with the residual substance which reduces indophenol at a velocity similar to that of ascorbic acid cannot at present be answered.

DISCUSSION.

If it be assumed, and there is a strong reason for doing so, that the residual indophenol-reducing capacity of the leucocytes is not due to ascorbic acid, their ascorbic acid content in " saturated " guinea-pigs is about 5 mg./100 g. of tissue. This figure approximates that of the muscles and bones of the body, which is about 4 mg./100 g. under the same conditions, and is well below those

of the so-called " selective " tissues such as the adrenal (150 mg./100 g.), the small intestine (15 mg./100 g.) and the liver (25 mg./100 g.) etc. Even if the total indophenol-reducing capacity of the leucocytes be attributed to ascorbic acid, they still cannot be placed among the tissues which are outstanding for their capacity of accumulating ascorbic acid. The question whether the ascorbic acid content of leucocytes circulating normally in the blood is of a similar order to that observed in the white cells in the exudates which were obtained by pathological stimulation must at present be left open.

At the same time it is of interest to compare the values obtained in this investigation with those of Stephens and Hawley. A full comparison is made difficult mainly because the last-mentioned workers have carried out their experiments on human subjects, and also because no detailed record of the intake of vitamin C during the period preceding the examination of the bloods is given by them. Stephens and Hawley make the general statement only that the values for whole blood and plasma corresponded to the previous vitamin C intake of the non-leukæmic subjects. No reference is made to the intake of the leukæmic patients. One outstanding feature of their results, nevertheless, deserves comment. The indophenol-reducing capacity of the leucocytes of the 11 leukæmic patients showed an average value equivalent to 25·1 mg. of ascorbic acid per 100 g. of cells, and in one case of lymphatic leukæmia a figure of 75 mg./100 g. was actually reached. The average value observed in the remaining 19 cases, which consisted of normal individuals and of patients suffering from a variety of other diseases, was only 12·5 mg./100 g. If these figures are accepted, it is difficult to believe that the markedly high reducing values observed in the leukæmic patients were due to accidental high intakes of vitamin C, especially as the analyses were carried out either before breakfast, or after a breakfast containing no appreciable quantities of vitamin C, and, therefore, several hours after the consumption of a meal, the vitamin C content of which could be responsible for a temporary rise in the ascorbic acid content of the blood-stream. Furthermore, as was seen above, the introduction of exceptionally high quantities of ascorbic acid into the blood-stream of " saturated " guinea-pigs was reflected only to a comparatively small extent in the indophenol-reducing capacity of the leucocytes, thus showing that normally a limit exists to the capacity of the leucocytes for accumulating ascorbic acid. These facts, taken in conjunction with the observation made in the present investigation that leucocytes may contain other reducing substances, rather favour the view that the rise in the indophenol-reducing capacity of the white cells in the leukæmic patients was in all probability not due to an increased content of ascorbic acid, but to the presence of another substance. This problem calls for further investigation.

SUMMARY.

Unlike most guinea-pig tissues which lose their capacity for reducing rapidly indophenol when the animals have been deprived of vitamin C for a few days, the leucocytes retain some of this capacity. In this respect they resemble the eye lens in which the residual reducing capacity was shown not to be due to the presence of ascorbic acid.

Trichloroacetic acid extracts of guinea-pig leucocytes give a nitroprusside reaction of about the same intensity as a trichloroacetic acid solution containing 7·5 mg. of glutathione per 100 ml. The velocity with which such a solution reduces indophenol is too slow to account for the residual rapid reducing capacity of the leucocytes of guinea-pigs, the tissues of which have been depleted of vitamin C.

The introduction of exceptionally high quantities of ascorbic acid into the blood-stream of guinea-pigs, "saturated" or "unsaturated" with ascorbic acid, produces comparatively little increase in the indophenol-reducing capacity of the leucocytes.

The bearing of the above results on Stephens and Hawley's observation of the high indophenol-reducing capacity of the leucocytes of leukæmic patients is discussed.

One of us (A. E. K.) is indebted to the Medical Research Council for a whole time grant.

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ERRATUM.

VOL. XIX, No. 3, PAGE 223, LINE 11.

For

β *Lr*/10 dose = 0·055 c.c.

read

β *Lr*/50 dose = 0·055 c.c.